Incorporation of Sequenced cDNA and Genomic Markers into the Soybean Genetic Map

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ABSTRACT

The soybean [*Glycine max* (L.) Merr.] expressed sequence tagged (EST) database is growing rapidly and promises to be a valuable resource for discovering agronomically important genes. Genetic maps featuring cDNA clones of known sequence and function are important because association of genes with phenotypes will increase understanding of the molecular mechanisms affecting valuable agronomic traits. Our objective is to place sequenced cDNA (EST) and genomic clones on an anchored soybean genetic map. The genetic mapping of these markers was conducted by standard restriction fragment length polymorphism (RFLP) techniques with an F_2 population of 149 individuals derived from a cross between two publicly available soybean genotypes cv. Noir 1 (PI 290136) and BARC-2 (Rj_4) (PI 547895). DNA sequences of mapped EST and genomic clones were compared with accessions in GenBank, and significant sequence similarities are reported. The ESTs were more likely than the genomic clones to have a significant similarity to a GenBank accession. Because the objective was to map ESTs and sequenced genomic clones, only the 24 linkage groups (1200 cM) containing the 39 mapped EST and sequenced genomic clone markers plus the four phenotypic traits root fluorescence (Fr_2), seed coat color (I), flower color (W_1) and nodulation response (Rj_4) were presented. Amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers were added to increase marker density. Simple sequence repeat (SSR) markers were included to align this map with other soybean maps. The population has been further advanced to develop a $F_{8:9}$ recombinant inbred line population available to researchers interested in associating the mapped cDNAs with quantitatively inherited traits.

The soybean expressed sequenced tag clone database is growing rapidly and promises to be a valuable resource for discovering genes of agronomic interest. ESTs are cDNA clones that have been partially or completely sequenced. Sequence similarity to known genes suggests gene function. These cDNA clones can be mapped by RFLP techniques just as cloned genomic fragments have been mapped. Mapping of cDNA clones places genes, some of known function and expression pattern, in regions of the genome that can be correlated with phenotypes. Therefore, association of cDNA clone markers with phenotypes can increase our understanding of biochemical pathways and mechanisms affecting agronomically important traits.

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Association of markers and phenotypes through genetic linkage has focused primarily on two goals: marker assisted selection and map-based cloning. Marker assisted selection increases the efficiency of tracking traits such as disease and pest resistance in cultivar development programs by significantly reducing the amount of screening with pathogens and pests. Map-based cloning utilizes genetic linkages between molecular markers and important traits to establish physical linkages and eventually to isolate genes controlling economically important phenotypes, such as resistance to nematodes (Cai et al., 1997; Ganal et al., 1995) and bacterial diseases (Mindrinos et al., 1994). Genetic maps containing EST and sequenced genomic clone markers are especially useful toward this end.

Soybean genetic linkage maps have been developed with several different kinds of markers. These include RFLP, RAPD, AFLP, and SSR markers (Cregan et al., 1999; Keim et al., 1992; Lark et al., 1993; Shoemaker and Olson, 1993; Shoemaker and Specht, 1995). Both RFLP and SSR markers have been used reliably to align maps from different populations. SSR markers have been especially valuable because they are highly polymorphic and utilize the ease and efficiency of the polymerase chain reaction (PCR) (Akkaya et al., 1992; Cregan et al., 1999).

EST and sequenced genomic clone markers also can be converted to PCR based markers by designing primers from their sequences. When primers derived from the sequences reveal useful polymorphisms with PCR assays, the loci they define are referred to as sequenced tagged sites (STS). Primers designed to detect single nucleotide differences between genotypes are called single nucleotide polymorphisms (SNPs). STS and SNP assays are designed to be conserved with regard to genome location and to be highly polymorphic. Therefore, ESTs, sequenced genomic clone markers, and their PCR-based derivatives are valuable tools for tracking and isolating genes controlling economically important phenotypic traits. Our objective is to place EST and sequenced genomic clone markers on an anchored soybean genetic map. Several of these markers represent fully sequenced and characterized cDNA clones of known identity, and others have high sequence similarity with genes of known function. The map includes SSR and phenotypic markers, so it can be compared

Abbreviations: AFLP, amplified fragment length polymorphism; bp, base pairs; cM, centimorgan; Da, dalton; EST, expressed sequence tagged; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, Restriction fragment length polymorphism; SSR, simple sequence repeat.

with and aligned with other soybean maps (Cregan et al., 1999) containing these markers.

MATERIALS AND METHODS

Population Development and Classification for Phenotypic Traits

A population of 149 F_2 individuals was derived from a cross pollination of soybean cv. Noir 1 (PI 290136; USDA Germplasm Collection, Urbana, IL) and BARC-2 (Rj_4) (PI 547895), a near-isogenic line derived from cv. Clark 63 and cv. Hill (Devine and O'Neill, 1986). Parental trait differences include soybean mosaic virus resistance, bacterial pustule resistance, nodulation (Rj_4), flower color (W_1), seed coat color (I) and root fluorescence (Fr_2) (Palmer and Kilen, 1987). The genotype of Noir 1 is rj_4 rj_4, w_1 w_1, i i, fr_2 fr_2. The genotype of BARC-2 (Rj_4) is Rj_4 Rj_4, W_1 W_1, I I, Fr_2 Fr_2. The dominant allele for each of the four traits exhibits complete dominance, so $F_{2:3}$ lines were evaluated to determine the genotype of each F_2 individual. Procedures for classification at the Rj_4 locus were described by Ude et al. (1999). Procedures for classification at the W_1 and Fr_2 loci were described by Devine et al. (1993). Procedures for classification of seed coat color were described by Weisemann et al. (1992). In addition to the above plant material, BSR 101 and PI 437654 were used to determine AFLP polymorphisms.

EST and RFLP Development

Soybean cv. Century cDNA clones and genomic clones were used to detect RFLP polymorphisms. Genomic clones were obtained from a genomic library made from *Pst*I digested DNA (Devine et al., 1993). Most cDNA clones were obtained from a library by means of mRNA from maturing soybean seed (Kalinski et al., 1990). The remainder of the cDNA clones was from a variety of other studies in which the cDNA clones were completely sequenced. The DNA sequence and characteristics of cDNA clones encoding cytosolic (Gebhardt et al., 1999) and plastidic aspartate aminotransferase (Wadsworth et al., 1993), aspartokinase homoserine dehydrogenase (Gebhardt et al., 1998), dihydrodipicolinic acid synthase (Silk et al., 1994), asparagine synthetase (Hughes et al., 1999), and a 34-kDa protein (Kalinski et al., 1990) were reported previously.

Although some of the cDNA clones used as RFLP probes previously had been completely sequenced and characterized, most of the cDNA and genomic clones were single pass sequenced from both 5′ and 3′ ends (Table 1). DNA was sequenced by dye-terminator cycle sequencing as described by the manufacturer (PE Applied Biosystems, Warrington, UK). The DNA sequence and corresponding amino acid sequence were compared with those in GenBank, EMBL, Gen pept, Swiss Prot, and PIR databases by the appropriate BLASTN and BLASTX algorithms.

Table 1. List of soybean cDNA and genomic clones used as RFLP markers and their GenBank accession numbers. Clone insert sizes and linkage groups to which the clones mapped also are included. Sequence identity comparisons are provided as the number of the accession to which the clone had the most similarity, the putative function of the accession, and the probability (*P*) value for the likelihood that the similarity of association is by random chance. If the clone was previously deposited in GenBank and characterized, the reference for the clone is given instead of the *P* value.

pBLT	GenBank Acc. No.	LG	Insert	Identity	With Acc. No.	*P* value (Ref)
			bp			
2	AW160133	I	cDNA (1500)	lipoxygenase	U37839	$8.9e^{-93}$
4	AW160134	N	cDNA (700)	unknown		
7	AQ872586	G8	genomic (1000)	mt. processing peptide	X66284	$1.1e^{-58}$
9	AW160135	G	cDNA (700)	unknown		
12	AW160136	O	cDNA (500)	unknown		
13	AW160137	B2	cDNA (1000)	heat shock protein	M99431	$1.3e^{-17}$
15	AQ872587	N	genomic (1050)	unknown		
16	AW160138	B2	cDNA (800)	HMG-y high-mobility group	X58246	$6.5e^{-116}$
17	AW160139	G1	cDNA (1000)	chalcone synthase 6	L03352	$9.6e^{-189}$
24	J05560	A2	cDNA (1350)	34-kDa seed thiol protease	J05560	Kalinski et al., 1990
25	AW160140	F	cDNA (800)	cytochrome 450 monooxygenase	AF022462	$2.2e^{-14}$
26	AW160141	O	cDNA (500)	ADR12 auxin down-regulated	X69641	$8.4e^{-137}$
27	AQ872588	O	genomic (800)	unknown		
28	AQ872589	G14	genomic (950)	unknown		
34	AW160142	C1	cDNA (700)	initiation factor	X79009	$3.6e^{-46}$
38	AQ872590	O	genomic (2500)	unknown		
42b	AQ903115	D2	genomic (600)	unknown		
44	AQ872591	G16	genomic (800)	unknown		
49	AQ872592	G17	genomic (1000)	unknown		
50	AF034210	G18	cDNA (1600)	cytosolic aspartate aminotransferase	AF034210	Gebhardt et al., 1998
51–2	AQ872593	K	genomic (1000)	unknown		
52	AQ872594	G5	genomic (2400)	unknown		
53	AQ872595	A2	genomic (1000)	unknown		
57	AQ872596	B2	genomic (2200)	sulfate transporter	AAF17685	$4.0e^{-48}$
62	AQ872597	N	genomic (2400)	unknown		
65	AF049706	A2	cDNA (450)	aspartokinase-homoserine dehydrogenase	AF049706-8	Gebhardt et al., 1999
68	L09792	D2	cDNA (1750)	plastidic aspartate aminotransferase	L09702	Wadsworth et al., 1993
71	AW160143	G21	cDNA (1800)	unknown		
72	AQ872598	A2	genomic (1600)	unknown		
74	AQ872599	G22	genomic (600)	unknown		
77	AQ872600	F	genomic (700)	unknown		
79	AW160144	C1	cDNA (900)	sulfhydryl endopeptidase	X51900	$2.8e^{-92}$
80	AW160145	O	cDNA (700)	unknown		
85	AQ903116	O	genomic (645)	transcription factor	AAD32870	$1.0e^{-04}$
86	AW160146	G4	cDNA (1100)	unknown		
93	AQ872601	G18	genomic (1800)	unknown		
94	L36436	G	cDNA (1500)	dihydrodipicolinic acid synthase	L36436	Silk et al., 1994
95	U77679	B2	cDNA (1989)	asparagine synthetase	U77679	Hughes et al., 1997

RFLP Hybridization

DNA was extracted from young leaves of parental lines and individual F_2 plants as described by Keim et al. (1988). Restriction enzymes used to digest genomic DNA included *Dra*I, *Ava*II, *Eco*RI, *Hae*III, *Hin*dIII, *Rsa*I, *Taq*I, and *Xba*I. DNA restriction fragments were separated by agarose gel electrophoresis and transferred to nylon membranes as described previously (Lin et al., 1996). The random priming reaction of DNA probes with [α-^{32}P] dCTP utilized the Amersham Pharmacia Biotech Inc. (Piscataway, NJ) Ready To Go DNA Labeling Beads according to manufacturers instructions. Hybridization, washing, and detection were conducted as described in Hughes et al. (1999). Kodak XomatAR X-ray film (Eastman Kodak Co., Rochester, NY) was exposed with intensifying screens to washed membranes wrapped in plastic wrap and developed.

Anchoring with SSR Markers

Most SSR markers were produced by PCR as described by Diwan and Cregan (1997) with fluorescently labeled primers. The parents of the population were screened with 52 SSR primer sets. A total of 36 of these primer sets produced polymorphisms and were used with the F_2 progeny for mapping. Reactions were multiplexed in the gel well and analyzed on an ABI373 DNA sequencer with GeneScan software (PE Applied Biosystems). Five additional SSR primer sets without fluorescent labels were used to anchor cDNA clone loci previously placed with known linkage groups. These SSR PCR products were separated on gels of 2.5 g of SFR agarose (Amresco, Solon, OH) per 100 mL TBE buffer and visualized by ethidium bromide staining.

RAPD and AFLP Markers

RAPD and AFLP markers were used to increase marker density. RAPD and AFLP techniques were described previously (Lin et al., 1996). AFLP markers are named according to the designation of Keim et al. (1997) for AFLP markers, listing the three *Eco*RI selective nucleotides, then the *Mse*I selective nucleotides, then size in base pairs. RAPD markers for this population were designated with the preface "rBLT." A listing of RAPD primer sequences and amplified polymorphic band sizes can be obtained from the corresponding author and will be made available at the web site http://bldg6.arsusda.gov/benlab (verified October 2, 2000).

Genetic Mapping

All molecular marker and phenotypic trait data were subjected to Chi-square analysis to test the goodness-of-fit of observed to expected ratios (1:2:1 or 3:1). Only loci fitting ($P = 0.05$) the Chi-square test were used in mapping procedures. The software program Mapmaker/Exp 3.0b was used to analyze the marker data to detect probable linkages at a LOD value of 3.0 (Lander et al., 1987). The "group," ''compare,'' ''try,'' and "ripple 6" commands were used to develop linkage groups and establish most likely gene order. SSR marker loci were used to associate linkage groups developed with this population and linkage groups of the soybean consensus map developed by Cregan et al. (1999). Where possible, linkage groups were named according to the designations of the consensus map (Cregan et al., 1999).

RESULTS AND DISCUSSION

Basic Map Structure

A total of 207 loci (156 presented) including 39 RFLP, four plant-phenotypic, 25 SSR, 105 AFLP, and 17 RAPD markers were grouped into 35 linkage groups covering approximately 1400 cM. Because our objective was to map EST and sequenced genomic clone markers, only the 24 linkage groups containing the 39 RFLP markers (20 cDNA and 19 genomic clones) and four plant-phenotypic loci are presented (Fig. 1). There are also 79 AFLP and 16 RAPD markers within these 24 linkage groups that cover approximately 1200 cM and are anchored with 18 SSR loci.

Use of SSR markers allows information to be translated among several populations segregating for more traits than for any single population. For example, the population used in this study segregates for some traits (soybean mosaic virus resistance, bacterial pustule resistance, nodulation response, and root fluorescence) not mapped in populations used to create the consensus map (Cregan et al., 1999). Thirty-six of the 52 SSR primer pairs used to screen the parental lines revealed polymorphisms between the two parents, and these SSR anchors enabled association of 20 of the linkage groups (15 presented) with those of other soybean genetic maps. The *I*, *Fr*$_2$, and *W*$_1$ loci are present on the consensus soybean map and also served as anchor loci. Where possible, our linkage groups having markers in common with those of Cregan et al. (1999) were given the same linkage group name. Linkage Groups B1, D1a/Q, and M from the soybean consensus map were not identified. Use of SSR markers to align maps will be especially important as researchers compare maps containing ESTs with maps featuring quantitative trait loci (QTL) to determine which ESTs represent genes that are candidates for genes controlling the quantitatively inherited traits.

Although the addition of PCR-based SSR markers was very valuable, PCR-based AFLP markers are not easily translated between soybean maps from different laboratories using different populations. We used some AFLP primers in common with Keim et al. (1997). Furthermore, we used BSR 101 and PI 437654, parents of the population used by Keim et al. (1997) to help us to identify AFLPs in common; however, we were not confident that AFLPs of similar size were actually identical between the two populations. Subtle differences in electrophoresis procedures and the use of different parental lines make alignment of AFLPs between the maps more difficult. Therefore, we did not make conclusions concerning the identities of the AFLPs mapped in this paper and those mapped by Keim et al. (1997). PCR-based RAPD markers were relatively noninformative; only 85 RAPD primers of 245 examined (35%) provided polymorphic bands.

Mapped ESTs and Genomic Clones

Thirty-eight of 63 polymorphic EST and sequenced genomic clones plus four phenotypic markers (*Fr*$_2$, *I*, *Rj*$_4$, and *W*$_1$) and marker NG24 were linked to other markers on this map (Table 1). These 38 clones were either sequenced previously and the results published, or the sequences were obtained in this work and submitted to GenBank. The accession numbers from GenBank

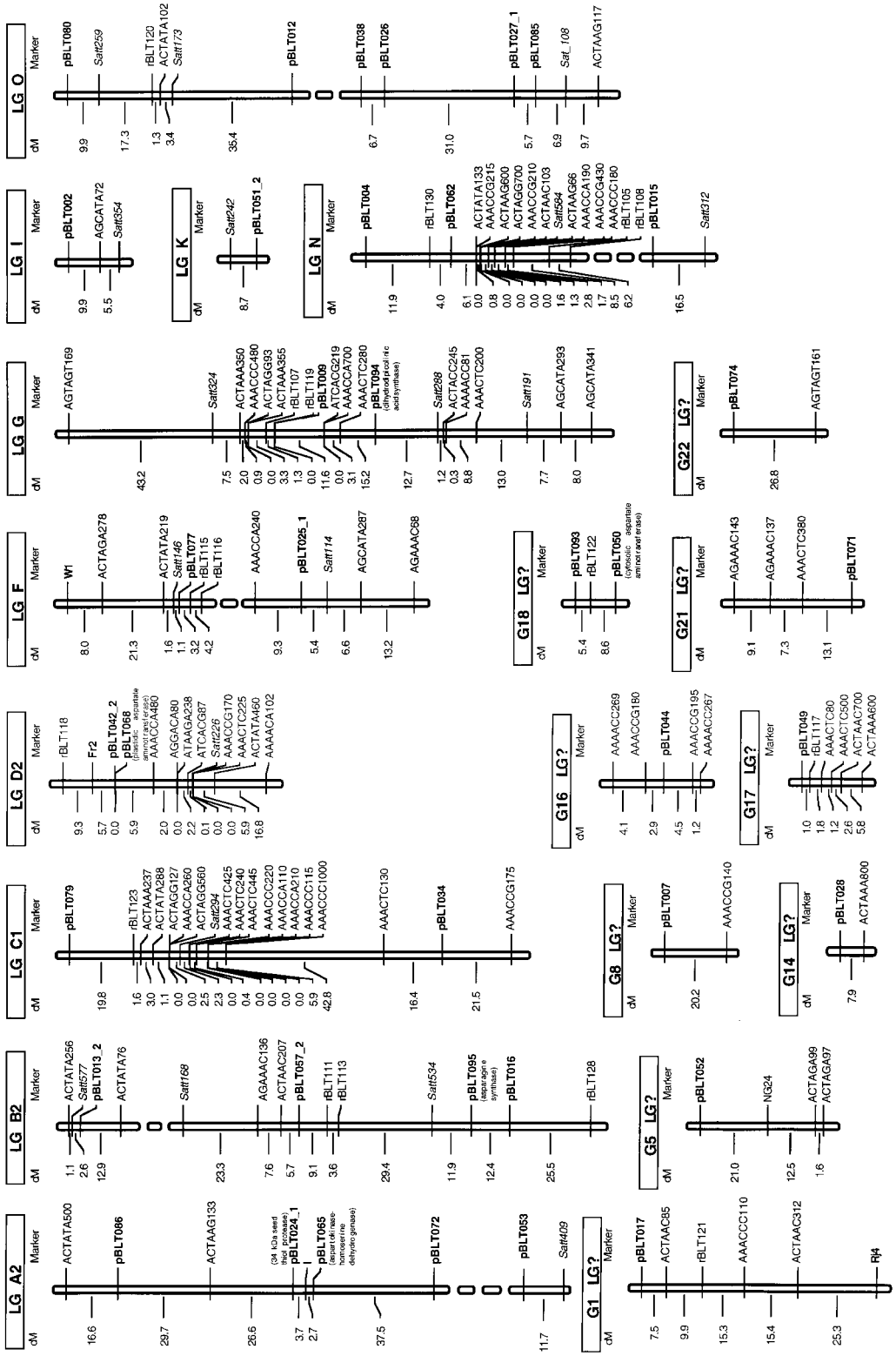

Fig. 1. Soybean genetic linkage map generated from a 149 individual F2 population derived from cv. Noir 1 (PI 290136) and cv. BARC-2 (Rj4) (PI 547895). Because this work emphasized placement of sequenced cDNA and genomic markers of known identity, only linkage groups containing these markers are represented. Six of the cDNA clones represent genes of known function, and the genes they represent are indicated in parentheses near the cDNA clone marker locus. Seven cDNA clones have detected multiple polymorphisms in this or other populations, and loci derived from those cDNA clones are designated to indicate the cDNA clone that identified them followed by the locus specification as published previously (for example pBLT24_1). SSR markers, in italics, were used to anchor linkage groups according to assignment in Cregan et al. (1999). AFLP markers are named according to Keim et al. (1997) (three *Eco*RI selective nucleotides, three *Mse*I selective nucleotides, size in base pairs). Dashed lines in linkage groups represent statistically unlinked regions of known linkage groups reported by Cregan et al. (1999).

are provided in Table 1. Interestingly, of the 200 plus clones from soybean cv. Century cDNA and genomic libraries screened, only a little over half identified polymorphisms with the eight restriction enzymes used. The 63 clones used for mapping were selected because the polymorphisms they detected were easiest to classify. Therefore, more of these are expected to link to other markers as marker density increases.

Previously, we reported the full cDNA sequences and some of the characteristics of six of the genes mapped in this study. These six cDNA clones encode cytoplasmic aspartate aminotransferase (pBLT50)(GenBank Acc. No. AF034210)(Ganal et al., 1995), aspartokinase-homoserine dehydrogenase (pBLT65)(GenBank Acc. No. AF049706)(Gebhardt et al., 1999; Matthews et al., 1998), plastidic aspartate aminotransferase (pBLT68) (GenBank Acc. No. L09792)(Wadsworth et al., 1993), a 34-kDa seed thioprotease (pBLT 24)(GenBank Acc. No. J05560)(Kalinski et al., 1990), dihydrodipicolinic acid synthase (pBLT 94)(GenBank Acc. No. L36436) (Silk et al., 1994), and asparagine synthetase (pBLT 95)(GenBank Acc. No. U77679)(Hughes et al., 1997). Only pBLT24 encoding a 34-kDa soybean seed protein and pBLT65 encoding aspartokinase-homoserine dehydrogenase have been mapped previously with knowledge of their sequence (Weisemann et al., 1992). Unknown cDNA and genomic clones for RFLP were single-pass sequenced at each end (except for marker NG24) and compared with computer databases for identity using BLASTX and BLASTN algorithms (Table 1). Identity is reported for best hits only if the probability of similarity association by random chance was equal to or less than e^{-3} for BLASTX and e^{-6} for BLASTN (Andrew Baxevanis, 1999, personal communication, see http://www.nhgri.nih.gov/COURSE99/, verified October 24, 2000). Analysis of ESTs revealed that some newly sequenced cDNA clones were of high identity to lipoxygenase (pBLT2) (GenBank Acc. No. AW160113) located on LG I, heat shock protein 83 (pBLT13)(GenBank Acc. No. AW160137) located on LG B2, HMG (*h*igh *m*obility *g*roup)-Y variant A protein (pBLT16) (GenBank Acc. No. AW160138) located on LG B2, chalcone synthase 6 (pBLT17)(GenBank Acc. No. AW160139) located on our LG G1, and ADR (auxin down-regulated) 12 (pBT26)(GenBank Acc. No. AW160141) located on LG O.

DNA sequencing and analysis revealed only three genomic RFLP probes with high identities to GenBank accessions. They were pBLT7, on our Linkage Group G8, with similarity to a portion of a gene encoding a mitochondrial processing peptide, pBLT85, on LG O, with similarity to a transcription factor, and pBLT 57, on LG B2, with similarity to a gene encoding a sulfate transporter protein. Although DNA sequence similarity searches will be more productive as DNA sequence databases grow, this will be truer for cDNA queries than for genomic queries, because of the number of ongoing EST projects, and because genomic queries contain DNA sequences that are not transcribed. The locations of aspartokinase-homoserine dehydrogenase (pBLT65) and the 34-kDa thiol protease (pBLT24) were already known to be located near the *I* locus (Matthews et al., 1998; Webb et al., 1995; Weisemann et al., 1992), which controls seed coat color. These markers also are near the Rhg_4 locus conferring resistance to the soybean cyst nematode race 3 (Webb et al., 1995; Weisemann et al., 1992). Analysis of the genome around the *I* locus indicates that more than one gene encoding aspartokinase homoserine-dehydrogenase is present in this region (Matthews et al., 1998). These clustered genes encoding aspartokinase-homoserine dehydrogenase possess highly similar DNA sequences. Similarly, in the same region near the *I* locus on Linkage Group A2 there are numerous chalcone synthase genes in a cluster (Todd and Vodkin, 1996). These include chalcone synthases 1, 3, and 4. Mutations in the *I* locus have been associated with a 2.3-kb *Hin*dIII fragment carrying the gene encoding chalcone synthase 4.

Although some gene family members were linked, others were not. The chalcone synthase we mapped is most similar to chalcone synthase 6 (pBLT17) and did not map near the chalcone synthase cluster on LG A2, but rather to our Linkage Group G1. Likewise, the cytosolic aspartate aminotransferase (pBLT 50) did not map to the same linkage group as the plastidic aspartate aminotransferase (pBLT68). We previously reported the linkage of pBLT68 and pBLT 42b with the Fr_2 locus (LG D2) (Devine et al., 1993), and have now determined that pBLT 68 encodes the plastidic aspartate aminotransferase. The cytosolic aspartate aminotransferase (pBLT50) is not located on LG D2 but rather on our Linkage Group G18. It is interesting to note that these aspartate aminotransferase genes are targeted to different cellular locations, map to different linkage groups, and do not have high DNA sequence identity with each other. Although aspartokinase-homoserine dehydrogenase and dihydrodipicolinic acid synthase are two enzymes in the pathway leading to synthesis of the aspartate family of amino acids, they are not genetically linked. While aspartokinase-homoserine dehydrogenase previously was mapped to LG A2, dihydrodipicolinic acid synthase mapped to LG G.

Important traits known to segregate, but not yet mapped, in this population include soybean mosaic virus resistance, and bacterial pustule resistance. In addition, a recombinant inbred line (RIL) population in the $F_{8:9}$ was developed from this cross for work with quantitatively inherited traits, including seed protein and oil content, yield, and maturity. To encourage future mapping using $F_{2:3}$ and $F_{8:9}$ progeny from this cross, the populations are available through T.E. Devine, and the F_2 mapping data are available through B.F. Matthews. Continued effort with this population may identify genes responsible for agronomically important qualitatively or quantitatively inherited traits through association between the traits and the cDNA clone markers present and added to this map.

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or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that also might be suitable.

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